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PRINCIPAL INVESTIGATOR: Richard B. Borgens
Director

CONTRACTING ORGANIZATION: Center for Paralysis Research
Purdue University
West Lafayette, Indiana

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Richard B. Borgens

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Center for Paralysis Research
School of Veterinary Medicine
Purdue University
West Lafayette, IN 47907-1244

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Applied DC electric fields have been demonstrated to induce a recovery of function following spinal cord injury (SCI). Originally this treatment for severe neurotrauma had been demonstrated in laboratory guinea pigs using a defined, and permanent, deficit in a sensorimotor reflex, the cutaneus trunci muscle reflex. This reflex is totally dependent on the integrity of long track white matter within the ventral spinal cord. Continuing studies have demonstrated the clinical utility of these procedures in cases of naturally produced, neurologically complete, SCI in dogs. A striking increase in recovery is noted when fully implantable stimulators are implanted within one month of the time of injury - but not if the application of a slowly oscillating DC field is delayed beyond this time. Evidence gained from laboratory rodents indicates the behavioral response is probably related to an electrically induced regeneration of nerve fibers within the transected spinal cord, and as well, perhaps a realignment of astrocytic cells that comprise CNS scar tissue.

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INTRODUCTION

STATEMENT OF THE PROBLEM

Severe spinal cord injury is well known to be one of the least tractable, but survivable, types of Central Nervous System (CNS) trauma. It affects at least 12,000 - 14,000 people annually. The most frequently occurring age at injury is 19 years and roughly 82% of the injured are male. Following motor vehicle accidents and acts of violence, severe SCI is an epidemic affecting the young involved in sports. Diving accidents contribute significantly (66%) to this category of injury, far eclipsing any other recreational sport (football, snow skiing, and surfing, approximately 6% each causing the greatest number of quadriplegics (Stover, 1986). Acute, severe, trauma to CNS soft tissue is a natural consequence of active military engagement - trauma in which there is no medical remedy that can prevent or alleviate long term care of military personnel - or even increase the quality of life for such seriously injured persons.

Support of this laboratory's research by the USAMRDC has allowed this laboratory to perfect techniques to modify the nervous system's response to trauma. Such techniques, while requiring surgery, are non-invasive with respect to CNS parenchyma, and could be applied in near-theater trauma care units. These techniques involve the use of applied DC electric fields, imposed across lesions by fully implantable indwelling stimulators. Originally these contracts also included support for experiments designed to facilitate both hard and soft tissue wound healing, however the response of the CNS to weak applied DC electric fields was much more striking. On the basis of USAMRDC reviewers comments, and on advice from Contract Officer Representatives, we have focused on CNS repair and regeneration exclusively in this - as well as the last - contract periods. Moreover, guidance by USARMDC scientific reviewers strongly suggested investigation into the biological basis for the

enhancement of CNS regeneration by applied DC fields as well as its cellular mechanisms of action (refer to Proposal Evaluation, AIBS Trauma Peer Review Panel to the USAMRDC, October 3, 1988). As in the past contract we have emphasized spinal cord injury models since we have a large supply of naturally injured canines available to us due to our association with the Department of Veterinary Clinical Sciences, and its Teaching Hospital. During this contract period, therefore, we have emphasized five areas of investigation relative to the use of applied DC fields as an acute treatment for transection of, as well as contusion to, the central neuroaxis of the adult mammal.

1. We have completed the first clinical test of applied electric fields in naturally produced canine paraplegia secondary to severe traumatic disc herniation (Hansen's type two) as well as in severe fracture/dislocation of the spine. The former is an excellent model for human paraplegia which usually results from severe contusion/distortion of the spinal cord resulting in profound central hemorrhagic necrosis of the parenchyma and subpial demyelination of surviving axons in the white matter (Borgens, 1992; Bunge et al., 1993).

2. We have further investigated the anatomical basis for the enhancement of CNS regeneration in a guinea pig spinal cord model - the cutaneous trunci muscle reflex (refer to section B) as well as the behavioral response to *delayed application of the electric field.*

3. We have investigated cellular mechanisms of action of the weak DC field in preventing nerve fiber - axonal - retrograde degeneration , and the relationship of the applied field to Ca^+ ion entry into injured axons. This Ca^+ influx is well known to produce axonal dissolution and degeneration (Schlaepfer, 1974 and 1983). Using *in*

vitro culture techniques, we have investigated if weak DC fields may influence other types of cells important in the biology of CNS injury and repair. Specifically the astrocyte. These cells undergo profound hyperplasia in response to wounding of the brain or spinal cord forming a dense cicatrix. This astroglia scar has been implicated as a barrier to CNS regeneration and repair (Reier et al., 1983).

4. Strategies aimed at facilitating nerve regeneration and behavioral recovery in the CNS must also recognize the problems associated with cell death in the spinal cord and brain secondary to hemorrhagic necrosis. Such has been the aim of investigators attempting to replenish nerve cells through fetal cell transplantation (Reier et al., 1992). We have pioneered the use of enteric neuron transplantation as an alternative method of nerve cell replacement. This methodology allows the recipient of the graft to be the donor of the replacement neurons (autogeneic neurotransplantation) and so circumvent the problem of tissue rejection mediated by the immune system.

5. Ancillary studies have also demonstrated that the efficacy of weak applied DC fields in facilitating CNS regeneration may be intimately related to the presence of organized DC voltage gradients that are expressed within the presumptive nervous systems in the vertebrate embryo. CNS development can be dramatically distorted if these endogenous electric fields are modified by a variety of means.

BACKGROUND OF PREVIOUS WORK

1. <u>Applied Electric Fields in Spinal Cord Injury: Preclinical Studies</u>

DC stimulators delivering regulated steady current have been developed and implanted into adult guinea pigs and rats following hemisection or contusion of the

spinal cord (Borgens et al., 1986a and 1986b, Wallace, et al., 1987). In hemisection studies an indwelling marker device was used allowing the identification of the exact plane of transection of intracellularly labeled nerve projections months postlesioning. Evaluation of the anatomy of ascending dorsal column axons and lateral tract axons in response to very weak applied fields was accomplished by anterograde filling of axons with horseradish peroxidase (HRP) (Borgens, et al., 1986a and 1986b). At very low field strength (ca. 1 μA total current; ca. 0.008 mV/mm) HRP labeled fibers could not be found within the glial scar which formed at the plane of transection. At intermediate field strengths (5 μA total current; ca. 0.04 mV/mm) ascending long tract axons had projected to the level of the lesion through the glial scar, but not through the scar into the rostral segment of the spinal cord. At field strengths of about 0.1 mV/mm (10 μA total current) fibers ramified throughout the glial scar and in a few cases deviated around the plane of the lesion into the rostral spinal cord segment (Borgens et al., 1986b). Some of these fibers possessed growth cones at their terminal ends within the rostral segment (Borgens et al., 1990). Labeled long tract axons were not detected within the glial scar or the immediate region of the transection in sham treated control animals, a characteristic feature of untreated lesions about two months post-injury. Retrograde HRP labeling caudal to "crush" lesions of descending projections in a rat model (Fehlings et al., 1988) demonstrated enhanced sparing of neurons by the applied field following injury. Increased numbers of HRP labeled cell bodies in the red nucleus, Raphes nucleus, lateral vestibular nucleus and the medulary reticular formation were observed in response to an applied DC field compared to sham-treated controls.

We have emphasized the axonal response to weak applied extracellular voltages. It is probable, however, that other cells such as astrocytes (whose hyperplasia leads to neurological scar formation (Reier et al., 1983) and macrophages (involved in secondary pathology following spinal cord injury) (Blight, 1985 and 1992)

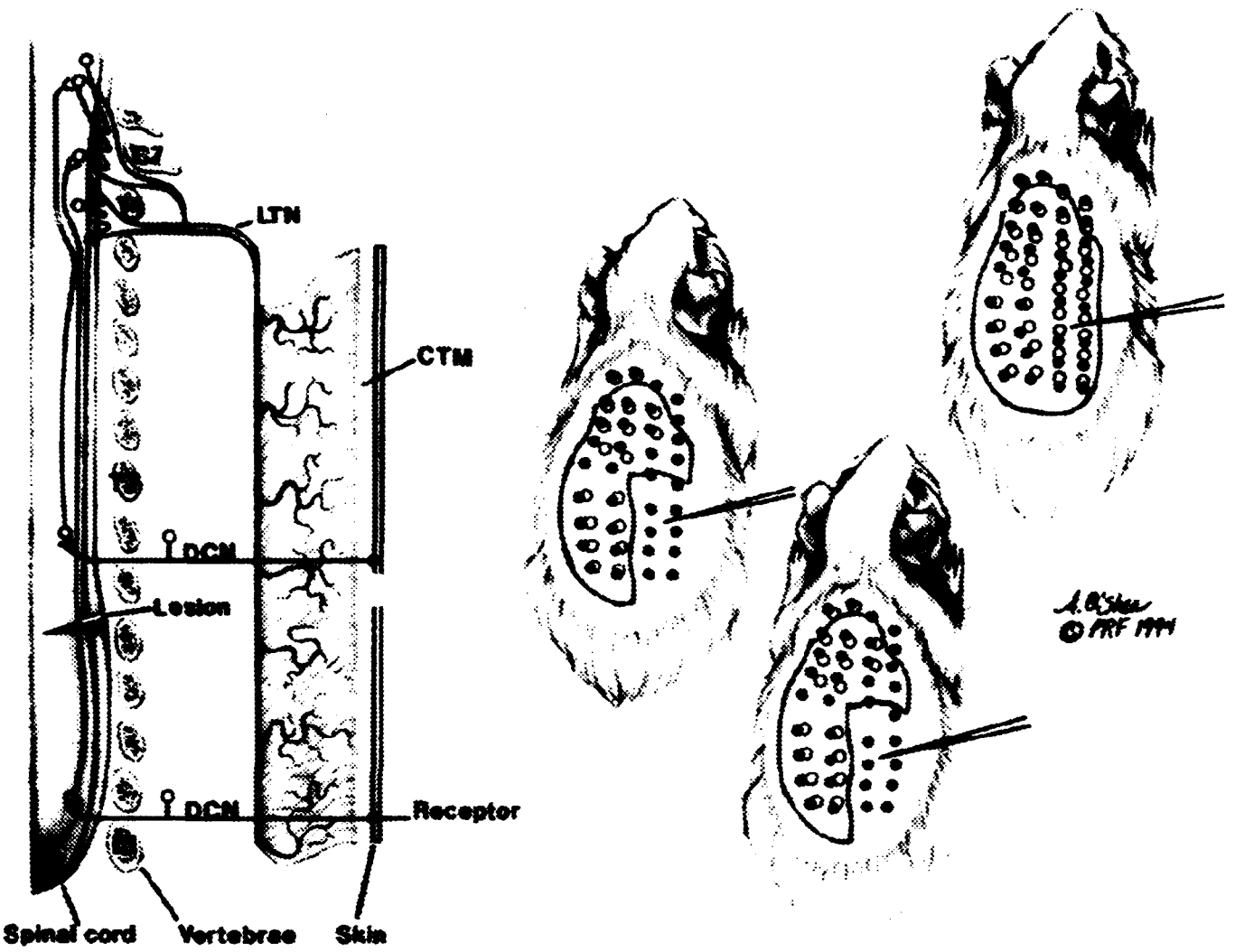
may also respond to steady extracellular voltages (Orida and Feldman, 1982), and be relevant to the anatomical responses to applied fields. This has been directly tested during the present contract period, and clearly demonstrates that GFAP positive astrocytes will indeed organize their cell body and processes perpendicular to the voltage gradient.

The behavioral consequence of electric field application has been tested in a novel guinea pig spinal cord injury model, the cutaneous trunci muscle (CTM) reflex. This is a complex sensorimotor behavior dependent on intact white matter of the ventrolateral funiculus of the spinal cord and normally observed as a "rippling" or "twitching" of the back skin in response to tactile stimulation (Blight et al., 1990) (Fig. 1). Complete lateral hemisection of ascending sensory CTM afferents produces a unilateral defect in the reflex. The behavioral loss following injury is observed as a permanent areflexia of back skin (below the vertebral level of the lesion, and on the same side). As expected, 100% of areflexic sham-treated control animals did not recover the reflex (Borgens et al., 1987, 1990). This was in contrast to a 25% recovery rate at the highest field strength tested (Borgens et al., 1987) when the cathode was situated rostral to the lesion. An application of reversed polarity appears to have no effect on recovery of the CTM reflex (Borgens et al., 1990). This might be the expected result remembering that axons only advance towards the cathode (negative pole) of an applied DC field on physiological substrates or *in vivo*. Though modest, this level of recovery is the first demonstration that a quantifiable, complex, sensorimotor reflex in the mammal - lost permanently after transection of the relevant spinal cord projections - can be recovered by any means. Less sensitive behavioral tests of function relying on ascending and descending white matter, but apparently responding to only one polarity of electric field application have been reported (Wallace et al., 1987; Fehlings, et al, 1988). These data as well suggest enhanced functional recovery but are not accompanied by an understanding of *how this may*

relate to the polarity of application. Since the CTM reflex only depends on ascending nerve fibers projecting to cervical motor units, one polarity of the applied field (rostrally negative) was found sufficient to recover the reflex. To be able to achieve a clinically useful stimulation regimen however, a means to theoretically influence both ascending and descending projections in the spinal cord had to be developed. A methodology called "oscillating field stimulation" (OFS) (Borgens and McCaig, 1989) born of a insightful observation by McCaig (1987) was developed to accomplish this. He noted that when neurites in culture experience an imposed DC field, growth responses (such as filapodia extension) towards the cathode may be immediate, while degenerative changes in neurites facing the anode take on the order of 45 minutes. The differing latency in the response to either polarity of the applied field may allow a window of opportunity where electric field imposition produces growth towards the cathode with relatively short exposure - but less than the time necessary to induce degenerative changes in fibers facing the anode. An implantable circuit which would reverse the polarity of the electric field application every 15 minutes was developed (Borgens and McCaig, 1989; Borgens et al., 1993b). Active OFS units and indistinguishable sham units were produced, coded, and used in a preliminary clinical trial of their effectiveness in cases of canine paraplegia (Borgens et al., 1993b).

Figure 1

Functional recovery of the CTM reflex following spinal cord hemisection in the adult guinea pig. *Left. Basic CTM reflex circuitry. The reflex behavior is observed as a twitching or rippling of the backskin in four legged animals in response to light tactile stimulation of the backskin. Touch receptors in the skin project afferents into the spinal cord at each vertebral level as components of the Dorsal Cutaneous Nerves (DCN). These synapse with long tract second order afferents that ascend the spinal cord within the ventrolateral funniculus on both right and left sides (only right side shown) projecting onto motor neurons contained in constellations at the thoracocervical level. Motor efferents leave the spinal cord at the brachial plexus and innervate the cutaneous trunci muscle of the skin as components of the lateral thoracic nerves (LTN). A hemisection of the spinal cord severing only the ventrolateral funniculus interrupts the ascending intramedullary spinal tract carrying CTM impulses and produces a permanent areflexia of backskin below the level of the hemisection and on the same side. Backskin above the level of the lesion and on the contralateral side is unaffected by the lesion, allowing the animal to serve as its own control. Complex skin movement in response to electrical or tactile stimulation by a sharp probe or calibrated monofilament can be quantitated by computer. A matrix of dots are tattooed on the animal's backskin. The skin is stimulated with a probe, and the movement of the dots (as the skin moves) is*



recorded on videotape. During this procedure a marker is used to circumscribe the region of skin where tactile stimulation produces skin movement - this is the receptive field. Using video image frame grab and graphics reconstruction techniques, a single image of the guinea pig is produced where a filled circle displays the position of the tattooed dot prior to stimulation and a open circle displays where it had moved following stimulation. Typically the skin "pulls" toward the point of tactile stimulation. The guinea pig in the center shows a typical response to unilateral (right) spinal cord hemisection at any time post lesion. Note that tactile stimulation below the level of the lesion and on the right side does not induce skin movement. This is the region of areflexia. Note as well the boundary of the skin's functional receptive field on the right side ends at about the level of the lesion and follows the midline caudally. The receptive field is normal on the left side for comparison. Following the application of rostrally negative, weak (ca. 0.4 mV/mm) DC electric field across the hemisection beginning at the time of lesioning, and lasting several weeks, a proportion of animals show functional recovery of the CTM reflex and receptive field (above right). In some cases functional recovery within this region is complete, in other cases, recovery is noted as islands of sensitive skin within the region of areflexia. Moreover, selective lesioning of DCNs in recovered animals eliminated the possibility that recovered areflexic regions were due to field-induced changes in peripheral innervation. The relevant changes underlying these recoveries of function occurred within the spinal cord. Sham - treated control guinea pigs do not recover and their receptive field appears similar to untreated animals (below right compare to center). Preliminary evidence suggests that a delay in the application of the field following hemisection fails to facilitate functional recovery.

2. Natural Produced Paraplegia in Dogs

Severe traumatic spinal cord injury in the canine produces the same clinical deficits observed in humans: loss of function, both motor and sensory, below the level of the lesion, spasticity, incontinence, and atrophy of the affected hind limbs. These problems produced by neurologically "complete" trauma show very little response to conventional medical and surgical intervention (including acute administration of steroids) (Borgens et al., 1993b; Toombs and Bauer, 1993). A clinical and neurological exam protocol was employed to isolate such severely injured animals from possible "incomplete" injuries presenting at the clinic, and to follow possible recoveries of function. This included tests for superficial and deep pain cognition, standard reflex testing (to identify upper motor neuron sequelae), a regimen of evoked potential tests (including Somatosensory Evoked Potential, Motor Evoked Potential, and Spinal Evoked Potential), ambulation, and tests for the loss of proprioceptive hind paw placing. To meet study criterion, a dog was required to be negative on all measures of neurological functioning (Borgens et al., 1993b). Study candidates were further subdivided on the basis of injury type and treatment time post injury. The acute traumatic disc herniation group (Hansen type II) (Borgens et al., 1993b; Toombs and Bauer, 1993) was quite interesting since this pathology is similar to most human spinal cord injury, namely severe compression of the spinal cord followed by central hemorrhagic necrosis (Bunge et al., 1993; Blight et al., 1991). One should not confuse this type of disc injury (which involves acute direct explosion of disc material into, sometimes laceration of, the spinal cord) in certain canine breeds with human "slipped" discs - which is rarely a spinal cord injury (refer to discussion in Borgens, 1992).

3. Applied Electric Fields Inhibit Traumatic Axonal Degeneration

As described in this text, it is clear that an applied DC field can induce a more robust nerve regeneration within the injured mammalian spinal cord (Borgens et al., 1986a, 1986b, 1990). We believe this control of nerve growth *in vivo* (ibid) or in vitro (Hinkle et al., 1981, Patel and Poo, 1982, McCaig 1986a, 1986b,1987) to be related to the role endogenous gradients of voltage play in the development of the nervous system (see section 5). The cellular mechanisms of action mediating galvanotaxis or galvanotropism in nerve fibers has been investigated by others (see review by Borgens and McCaig, 1989), however the acute retrograde degeneration of nerve fibers has not been analyzed. When a nerve fiber has been severed or contused, a very strong inwardly directed ionic current is produced, entering the lesion, and generated by the inwardly negative resting potential of the intact surrounding membrane (Borgens et al., 1980). About 30% of the ionic composition of this injury current is Ca^+, which is known to cause a destruction of the cytoplasm through depolymerization of the cytoskeletal proteins and other autocatalytic Ca^+ mediated events (Schlaepfer, 1974 and 1983). Using the descending giant reticulospinal axons of the lamprey ammocoete, Roederer et al., 1983 found that a distally negative applied extracellular voltage reduced the extent of axonal retrograde degeneration (or axonal dieback) compared to control fibers, while a distally positive extracellular voltage gradient enhanced the extent of retrograde degeneration of tnese identifiable fibers (Figure 2). We have long suspected that the mechanisms of action of applied fields on the *acute* responses of nerve fibers involves the production of a "bucking voltage" within the nerve fibers terminal end that may reduce the magnitude of the inwardly directed current of injury. If this is so, one would suspect that a distally negative voltage would lessen the influx of Ca^+ into the end of the fiber, while a distally positive extracellular voltage would enhance Ca^+ entry. In collaboration with K.R. Robinson's laboratory, we have tested this notion by following the concentration of Ca^+ within

severed lamprey giant axons with the intracellular Ca^{+} probe, Fura 2. (Strautman et al., 1986) We have learned that distally negative extracellular fields reduce terminal accumulations of free Ca^{++} - this being associated with decreased axonal dieback. Conversely, distally positive imposed fields increase Ca^{++} in the injured tip, producing graded axonal dieback relative to control axons (Strautman et al., 1990).

Figure 2

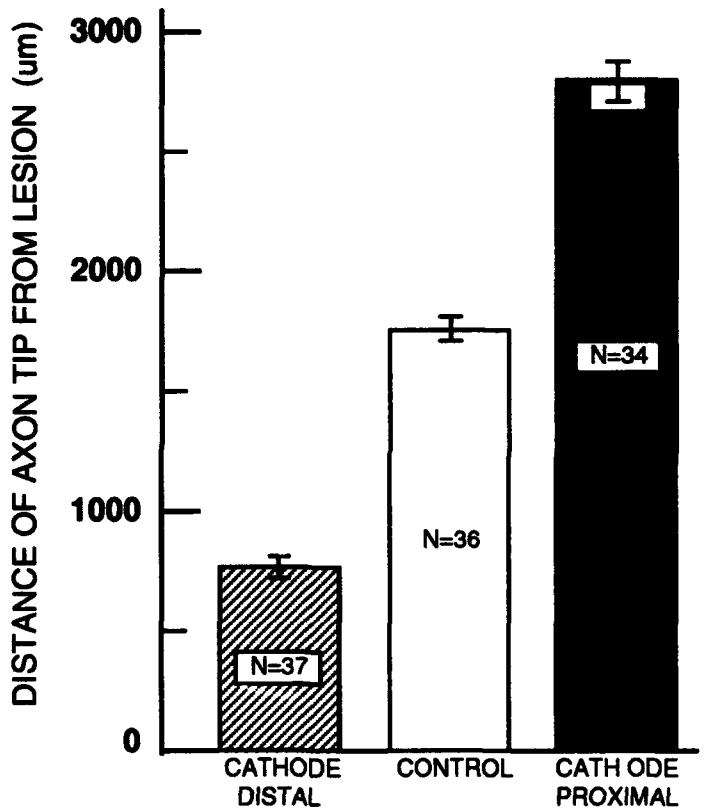


Diagram showing the mean and SEM of axon dieback in the three treatment groups. N is the number of intracellularly marked nerve fibers. (from Roederer et al., 1983)

4. <u>The Astrocyte as a Cellular Target of the Applied Field in Spinal Cord Injury</u>

Most of the narrative in this text, and the original proposal, emphasized the known ways that extracellular voltages affect nerve fiber dieback and regeneration following acute trauma. Injuries to the brain and spinal cord also involve the participation of

another race of cells - glia - which are crucial to the eventual possibility of functional recovery. In non-mammalian vertebrates, especially those that regenerate their spinal cords such as fish (Bernstein, 1968), salamanders (Stensass, 1983) and the tails of certain reptiles (Simpson, 1983), glial cells appear to both provide fasciculation pathways facilitating the growth of axons across or through the lesion as well as provide a source of stem cells for the differentiation of new neurons (Simpson, 1983). In the mammal, astrocytes undergo a profound hypertrophy, forming a dense cellular barrier to nerve regeneration (Reier et al., 1983). Given the importance of cells of glial lineage such as appendyma (in the non-mammalian vertebrates) and astrocytes, we have tested these cells to determine if they respond in a predictable manner to applied voltages. If they do, this might suggest another manner in which applied DC voltages might affect the biology of mammalian CNS injury leading to functional recovery.

5. *Endogenous fields guide the development of the nervous system*

It has long been suspected that endogenous steady ionic currents and natural voltage gradients are controlling factors in the development of animals and plants (Lund, 1947; Jaffe and Nuccitelli, 1977; Jaffe, 1980, 1981). The evidence supporting the critical role of such endogenous voltages to development is largely circumstantial however. Only recently have new studies provided direct evidence for a controlling role of endogenous voltages in vertebrate morphogenesis, particularly the early nervous system. It is also clear that anatomical changes and behavioral recovery can accompany the application of weak steady voltages to traumatic injury of the nervous system. The experiments suggesting that natural gradients of voltage may help control early neural development in the vertebrate embryo also provide insights into the effective use of imposed electric fields as a new clinical treatment for spinal cord injury.

The integument of most animals, including humans, maintains a steady potential difference across itself on the order of 30 - 60 mV (Borgens, 1982; Vanable,

1989; Barker et al., 1982). This inwardly positive voltage is produced by specific Na^+ entry through apical membranes of the superficial layer of epidermis coupled to the energy dependent pumping of this ion into the intracellular space by basal membranes (Kirschner, 1973; Hamilton and Eaton, 1985). Since the apical domains are sealed by tight junctions (limiting or eliminating Na^+ movement in the reverse direction) (Simmons, 1990) an electrochemical gradient is formed polarizing the epidermal syncytium. The Na^+ channel within apical membranes is unique, and not to be confused with the typical voltage dependent Na^+ channel helping to support the resting potential of cell membranes (Sariban-Sohraby and Benos, 1986). In epithelia, the apical Na^+ channel is sensitive to blockade by specific agents such as amiloride benzamil, the methyl ester of lysine (serving to collapse the potential) (Kirschner, 1973) or novobiocin (which facilitates Na^+ movement through apical membranes) which serves to increase the transepithelial potential (TEP) (Johnston and Hoshiko, 1971; Rick et al., 1988; Shi and Borgens, 1994).

In the axolotl neurulae, a gradient of 10 - 20 mV/mm is observed beneath the neural plate (Metcalf et al., 1994), positive at the head with respect to the tail. Over distances of 400-800 μm, this gradient may reach 60-80 mV/mm. A more shallow voltage gradient is observed in the transverse plane of axolotl neurulae associated with mirror image outwardly directed currents at the lateral walls of the left and right neural folds (Metcalf et al., 1994). The subectodermal lateral margins of the folds are thus negative with respect to adjacent neural plate or flank ectoderm. The rostral/caudal gradients of voltage beneath the neural plate and the neural fold become apparent at the beginning of neurulation and the neural fold outcurrents disappear at its climax just prior to neural fold fusion (Metcalf et al., 1994). These extracellular voltages are driven by the Na^+ dependent TEP of the ectoderm described above. In summary, amphibian neurulae are electrically polarized in the rostral/caudal dimension, and right and left transverse dimension, but only during the pageant of

neurulation. This is in spite of the fact that gastrulae maintain a TEP across the ectoderm as well (Metcalf and Borgens, 1994) There is now direct evidence supporting the critical nature of these ionic currents and internal voltages to the emerging ground plan of the early nervous system in these species.

Figure 3 Ionic Currents Traverse Amphibian Embryos

Spatially organized patterns of a steady ionic current are driven through the urodele and anuran embryo during neurulation. Current is driven out the lateral margins of the neural folds and out of the blastopore, returning through the general body surface. The battery driving this current out of the embryo is the inwardly positive transepithelial potential (ca. 20 - 50 mV) of embryonic ectoderm (Metcalf et al., 1994). Since the direction of current flow in biological systems is defined as the direction in which positive charges move, net ionic current leaks out of ectodermal regions of low electrical resistance. This current can be detected non-invasively with a vibrating electrode positioned near, but not touching, the embryo (Jaffe and Nuccitelli, 1974; Metcalf et al., 1994).

BODY

APPROACH, METHODOLOGY, AND RESULTS

1. Indwelling Stimulator Fabrication: Guinea pig

The DC stimulators use two 3-volt lithium dioxide cells (Ray-O-Vac #BR 1225 or equivalent) connected in series. Silver epoxy is used to attach leads to the cells or the cells to each other if stacked. A constant-current source (LM 334, National Semiconductor) and two resistors are soldered together and attached to the battery with silver epoxy. One resistor, R_{set}, is used to set the current level to any value between 1 μA and 10 mA. This resistor is connected between pins 2 and 3 of the LM 334. The other resistor, Rm is a 1,000 Ω resistor, placed in series with the output, allowing the current to be monitored by measuring the voltage drop across the resistor. (One microampere of current will produce one millivolt of potential). Monitoring leads are soldered on either side of R_m. The monitoring leads are made of a highly flexible silicone insulated multistrand wire (AS-155-36, Cooner Electronics, Chatsworth, CA) and can either be left long and exteriorized percutaneously or cut short and left inside the body. The electrodes are of the "wick" or salt bridge design as previously used in references Borgens et al., 1986b and 1987. These silastic tubes (filled with agar/ringer slurry) are slipped over Ag/AgCl terminals attached to either pole of the circuit by Ag conductive epoxy and this joint sealed with elastomer. The use of "wick" stimulating electrodes eliminates electrode product contamination of the target tissue and further avoids problems of interpretation of possible subtle anatomical results. The unit delivers 50 μA regulated current for about 1 month.

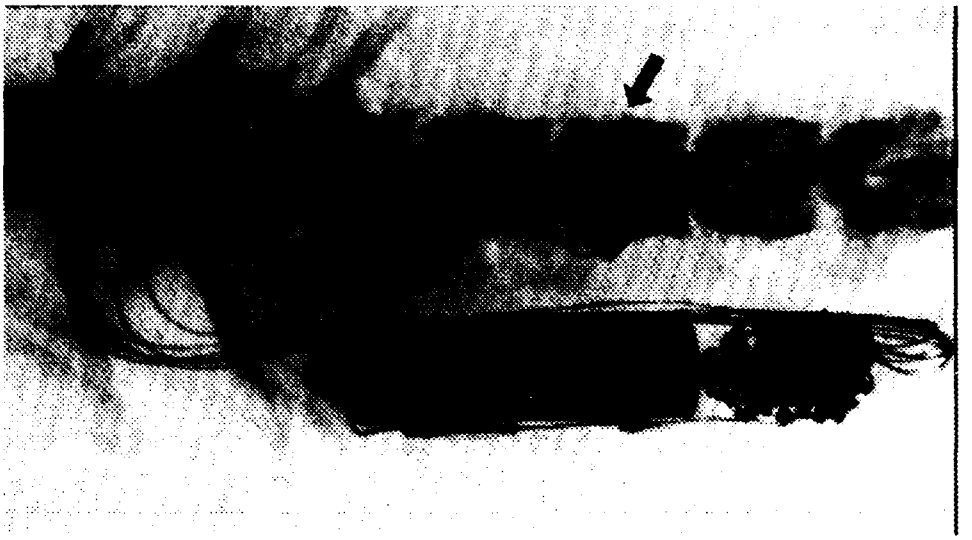
2. General Surgical Procedure: Guinea Pig

Adult female guinea pigs of the Hartley strain (400-600g body weight) are used in all of our studies. Animals are anesthetized for surgery by intramuscular injection of a mixture of 60 mg/kg ketamine HCl, 0.6 mg/kg acepromazine maleate, and 12 mg/kg xylazine. Sterile procedures are maintained throughout, with the assistance of a surgery technician. A 2 cm midline dorsal thoracic incision is made to expose the vertebral column at T11-13. A one segment laminectomy is performed with rongeurs. Either a dorsal hemisection (for Dorsal column and corticospinal tract study) or right lateral hemisection (for CTM study) is carried out with a small cutting device fashioned from a razor blade and subsequently confirmed with an electrolytically sharpened insect pin passed through the lesion. Stimulator units designed for subcutaneous placement are located beneath the back skin and stimulating electrodes routed to positions approximately 2 vertebral segments on either side of the hemisection. Here a partial laminectomy is performed, exposing the spinal cord for a distance of about 3 mm between two vertebrae. Stimulating electrodes are located within these partial laminectomies and sutured to the dorsal fascia so that the electrodes are near the exposed cord (1-2 mm) but do not touch it. Sham and operative stimulator units are identical in appearance and coded by the fabricator, thus the surgeon is blinded to the application at the time of implantation.

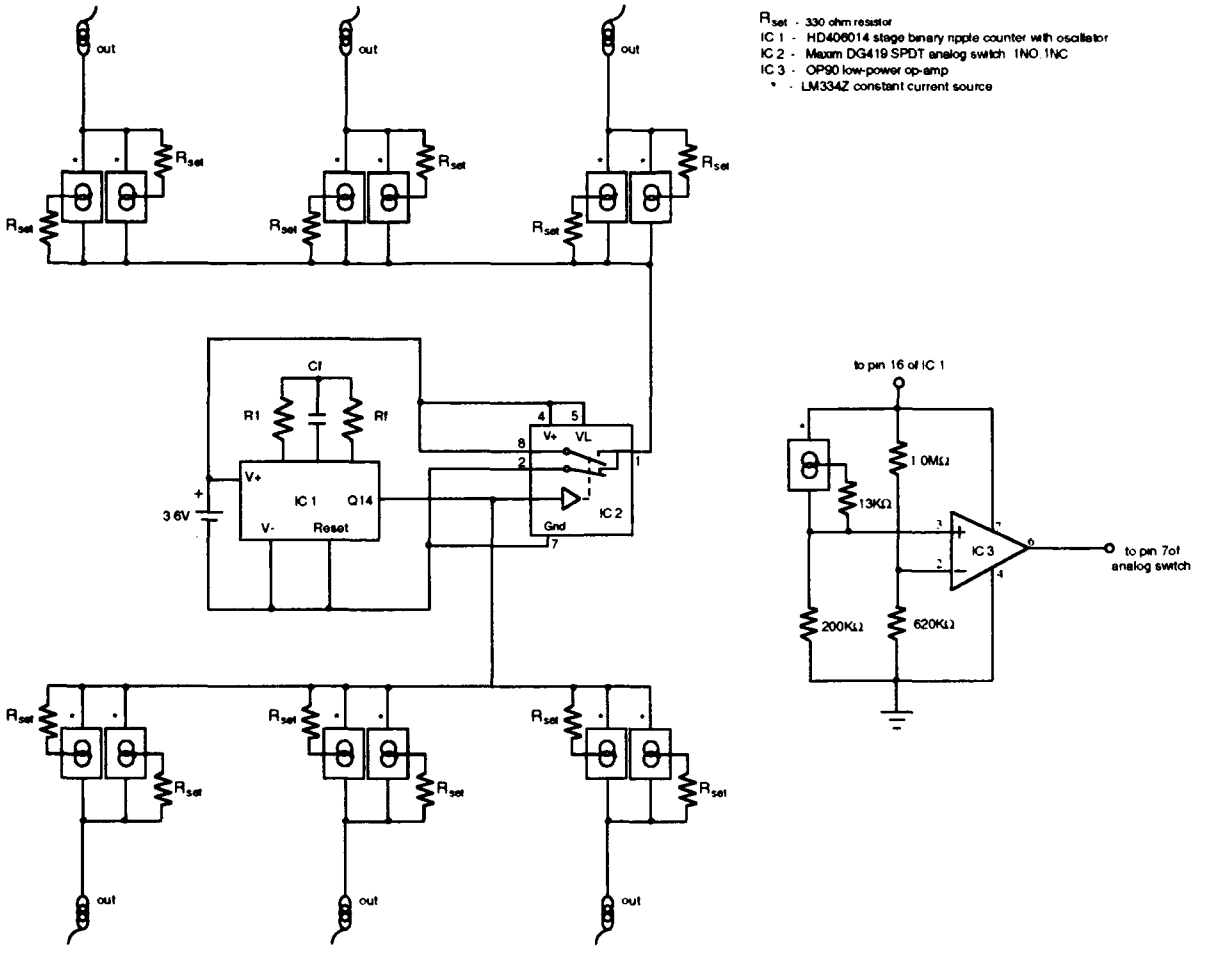
The wound is closed in layers, using 5-0 monofilament, following the injury and implantation of the stimulator. The animal is allowed to recover from anesthesia, with maintenance of core temperature on a heating blanket, and is kept for various times. We employ specialized care to prevent complication from urinary retention, pressure sores, and autophagy.

3. General Surgical Procedure: Clinical Cases of Canine Paraplegia

Following admission to the study, dogs are scheduled for immediate surgery for decompression of the spinal cord and implantation of a coded stimulator unit. The most current and comprehensive veterinary management of the spinal cord injury will be administered to all dogs. Prior to surgery, all dogs will be given a prophylactic dose of 30 mg/kg methylprednisolone sodium succinate (IV). General anesthesia will be induced with sodium pentothal (4 mg/kg IV), and maintained with a mixture of oxygen and gaseous anesthetic agents (isofluorane or halothane). The spinal cord at the site of injury, identified radiologically, will be exposed by hemilaminectomy, extended rostrally and caudally from the site of injury to the extent of cord swelling or the presence of disc material in the vertebral canal. Disc material will be removed from the spinal cord and canal. The exposed spinal cord will be covered with an autogenous fat graft and the uninsulated ends of the OFS stimulator (Figures 4 and 5) electrode leads will be attached by suture to paravertebral muscle at either end of the laminectomy opening, usually 5-10 mm above the dorsal surface of the spinal cord (Figure 4). The orientation and placement of the electrodes later will be evaluated from radiographs. The insulated electrode leads will be routed through the muscle layers during closure, and the stimulator package itself implanted in a subcutaneous pocket opened by blunt dissection to one side of the midline incision. Standard surgical procedures will be followed for skin closure and post-operative management.



Figures 4 and 5 Circuit characteristic of 6 lead, OFS stimulator (below) and radiograph of unit implanted in canine paraplegic. Note trio of electrodes tethered equidistant from each other and cranial and caudal to the lesion



4. General Surgical Procedure: Enteric Neuron Neurotransplantation

i. Donor Tissue

Sprague-Dawley rats (200g) provide enteric tissue for the grafts. Animals are fasted for 28 h, and deeply anesthetized by an overdose of nembutal (60 mg/kg). Following cardiac arrest, portions of the jejunum and ileum are removed and thoroughly rinsed with several changes of sterile Hank's balanced salt solution (HBSS). The small intestine is cut into approximately 1-cm-long segments and stored in HBSS on ice. A plastic pipette tip was slipped into each segment of the small intestine to stretch the contracted muscle layers and facilitate removal of the myenteric plexus attached to smooth muscle fibers of the longitudinal muscle layer (myenteric plexus and muscle, MPM). This is done with two fine-tipped forceps to both grasp the intestinal segment and to tease off the MPM which is collected in small vials containing ice-cold HBSS. Pieces of muscle with attached ganglia were incubated in dispase (1 drop per ml of HBSS) for 15-20 min. The enzyme is removed and enzyme digestion stopped by suspending the partially dissociated tissue pieces in complete culture medium containing 15% horse serum. Mechanical dissociation is carried out in medium using fire polished glass pipettes of decreasing diameters. The resulting tissue fragment suspension is placed into 35-mm culture dishes at 1 ml per dish. Explant labeling rhodamine-conjugated microbeads are added to the cultures - 0.5 µl per dish. After 4 h incubation, the rhodamine-containing medium is exchanged for fresh growth medium. The medium is changed again prior to tissue implantation in order to dilute the superficially adhering microbeads. Alternatively, the method of Powley and Berthoud (1991) was employed to label enteric neuron *in situ* with FluoroGold three to five days prior to their isolation.

ii. Explant culture

Explants are grown in 35-mm tissue culture dishes coated with rat tail collagen. To faciliteate subsequent histological processing, several cultures were grown in eight-well substrates. Growth medium consisted of RPMI-1640 liquid medium (Whittaker Bioproducts, Inc., Walkersville, MD) supplemented with 10% heat-inactivated horse serum and 10% fetal bovine serum (Hazelton Biologics, Inc., Lenexa, KS). The growth medium was exchanged on alternate days. For cell typing, explant cultures were maintained for ten days *in vitro*.

iii. Spinal cord injury and graft replacement

Rats are anesthetized with an i.p. injection of ketamine/xylazine (20 mg ketamine plus 2.2 mg xylazine per 200 g body weight) and prepared for aseptic surgery. The dorsal aspect of the lower thoracic and upper lumbar vertebral column is surgically exposed and a laminectomy performed. The dura was carefully removed with No. 5 Dupont forceps. A fluid-filled glass probe (0.8 mm diameter) attached via polyethylene tubing to a 50-µl Hamilton syringe is used to pick up the graft by aspiration. Following insertion of the hand-held probe into the right dorsolateral region of the exposed cord (which generates an injury injection track), the transplant is pressure injected. Graft replacement was visually observed to be complete in all cases.

We have shown that enteric neurons, removed from the myoenteric plexus, disassociated, and cultured, can serve as such a possible alternative to fetal cell transplants (Jaeger et al., 1993). As described above, these cells were dissagregated by enzymatic and structural disassociation from the myoenteric plexus of fully adult rats, settled in culture, and labeled with rhodamine complexed microspheres. This

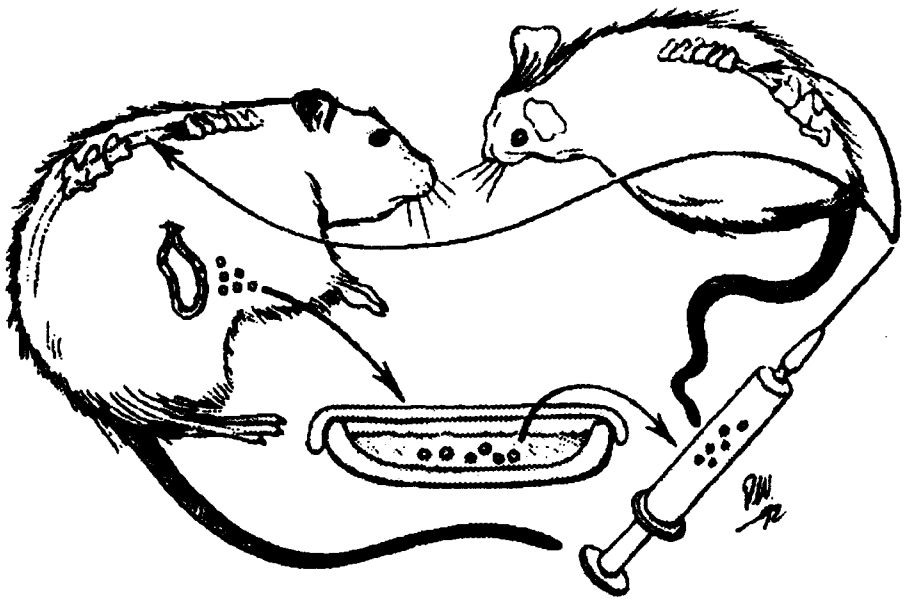


Figure 6 Autogeneic and allogeneic transplants to the rat spinal cord. Enteric tissues containing neurons are gently scraped from the outside lining of the intestine (on the rat on the left). These are placed in culture, carefully broken down into tissue fragments by gentle enzyme' treatment and mechanical disruption, and marked with a fluorescent label. These labeled fragments form the substance of the "graft" which is then injected into the spinal cord several days later. When the graft is injected into the same rat that provided the enteric tissues (the one on the left) this procedure is called an autogeneic graft. When injected into a different animal (on the right), the procedure is called an allogeneic graft.

intracellular marker later allowed a facile determination of host cells from graft cells following transplantation to the adult rat spinal cord. We performed these first tests using allogeneic grafting techniques, where the cellular material from two to three rats was transplanted to over 20 different individuals. The eventual degradation of the graft via the slow immune response of the rat was a further test that the tissue of the graft was indeed donated to this site. This was emphasized by the clustering of ed-1 positive macrophages at the graft's boundaries (Jaeger et al., 1993). Electron microscope evaluation also demonstrated the presence of growth cones on the nerve terminals of processes within the graft . We could not identify, however, if the neuronal source of these terminals was of graft or host origin. It was clear then from autogeneic grafting procedures that enteric neurons, both intracellularly labeled and acetyl

cholinesterase positive, could be removed from the gut of the adult mammal, and translocated to the spinal cord where the graft persisted until destroyed by tissue rejection. In unpublished work, we have learned that the same technique works equally well when the graft is moved back from culture to the donor animal's spinal cord. In such autogeneic grafts, the neuron population remains viable indefinitely (we have followed these transplants for up to nine moths post surgery). In addition, we have preliminary evidence which requires corroboration for the following: a) regenerating nerve processes from outside the graft penetrate its interior and form connections. We have no evidence yet that growing processes from inside the graft leave it to form connections with the host tissues and b) inwardly growing nerve processes regenerate through tunnels formed of glia which facilitate their penetration into the graft across its limiting boundary tissues (Jaeger, 1993).

5. Behavioral Evaluation: Guinea Pig Spinal Cord Injury

i. Behavioral Recovery in the CTM reflex by applied DC Fields.

Since CTM receptive field functioning is dependent on ascending units within the spinal cord white matter the *cathode was affixed in paravertebral musculature near but not touching the spinal cord ca. 2 cm rostral to a right lateral hemisection*, the anode was similarly attached but caudal to the lesion. Implanted 50 μA units employing wick electrodes were first tested. The responses to this application were compared to a sham treated control group (Borgens et al., 1987 and 1990). In 25% of experimental animals the CTM reflex had recovered, i.e., a restoration of the areflexic receptive field was noted about 56 days post hemisection and stimulator implantation (Borgens et al., 1987). Intact receptive fields ipsilateral to, and above the lesion were unchanged as were intact contralateral receptive fields (left side). The areflexic region in all control (sham treated) animals did not recover and remained unresponsive. The quantification of the reflex utilized a system where a matrix of permanently tattooed

marks on the shaved back skin provides an index of movement when a complete routine of tactile stimulation was videotaped and analyzed (refer to methods and appendix materials) (Borgens et al., 1987 and 1990) as well as electromyographic techniques. Delayed application of the field (approximately 3 months post hemisection) does not lead to a functional recovery (Borgens et al., 1993a). To our knowledge this is the only example of a recovery of function in a quantifiable and otherwise permanent behavioral deficit in the adult mammal produced by *select spinal cord lesion utilizing any treatment.*

ii. CTM Behavioral Analysis: Electromyography and Videographic Analysis

To visualize and record skin movements in response to tactile or electrical stimulation, we first shave the back of the animal and mark the skin with a matrix of black dots (using a permanent tattoo). These dots move during skin contraction. To analyze such movements we use a commercially available (Magic) video-image digitizer attached to a Macintosh SE computer. This allows succeeding frames of the video recordings to be digitized in a dot matrix pattern. Each digitized image is then transferred to a graphics program (Superpaint) which allows superimposition of a succession of images. Finally, the image from the most extreme point of skin contraction can be superimposed on the image preceding the onset of contraction to give a complete vectorial representation of the movement of the skin. This system allows us to analyze the movements in great detail and reveals important features of the process of functional recovery in the CTM system, both with regard to spatial distribution and timing (see appendix materials) (Borgens et al., 1990, 1993a and Blight et al., 1990). Electromyographic recordings of skin contraction are performed on animals lightly anaesthetized with sodium pentobarbital. EMG's are recorded from subdermal wire electrodes and are amplified with a Grass P 15 D preamplifier and displayed on a digital oscilloscope (Nicolet #310). Permanent records are saved to a

floppy disk and later plotted on a Hewlett Packard Color Pro Plotter. Examples of such physiological recording of the CTM reflex (Borgens et al., 1987, 1990 and Blight et al., 1990). The illustration in Figure 1, Introduction, demonstrates this type of evaluation.

6. Behavioral Evaluation: Canine Paraplegia

Since the resolution of behavioral deficits in a clinical injury might involve interaction of the applied field with ascending and descending tracts, we developed a methodology called "oscillating field stimulation" (OFS) born of an interesting observation by McCaig, 1987. He noted that when neurites in culture experience an imposed DC field, growth responses towards the cathode may be immediate, while degenerative changes in neurites facing the anode take on the order of 45 minutes. The differing latency in the response to either polarity of the applied field may allow a window of opportunity where field imposition produces growth towards the cathode, with relatively short exposure - but less than the time necessary to induce degenerative changes at the anode. We developed an implantable circuit which would reverse the polarity of application every 15 minutes. The current regulating and timing circuitry to accomplish this have been published elsewhere (see Figures 4 and 5). Active OFS units and indistinguishable "sham" units were produced in our laboratory, coded and provided the clinic for use in a randomized, blinded trial of their effectiveness in naturally produced canine paraplegia. All neurological exams were video taped at prespecified times: prior to surgery, just post-surgery, at the 6-8 week and 6 month recheck. Some neurological evaluations (deep pain and ambulation) were scored at the end of the experiment by comparison of video records for all dogs in the trial by a panel of investigators blinded to their status. The general conduct of this trial and the results are described in Borgens et al., 1993b: emphasizing the acute (less than 1 month post injury) compression injury group. Briefly our results were; 1) a strong trend for recovery of function *in all neurological categories* was evident in the

experimental group *with no reverse trends*, compared to the sham-treated canines. 2) Only 15% of OFS treated dogs failed to recover in some category of evaluation compared to 60% of the sham-treated controls. 3) Approximately one-third of the OFS treated dogs recovered in all 4 categories of behavioral evaluation - *none of the sham treated animals did* (see figure 7, below). 4) The combined neurological score was significantly different between these groups at 8 weeks ($p < 0.033$) and at the 6 month ($p < 0.035$) recheck period.

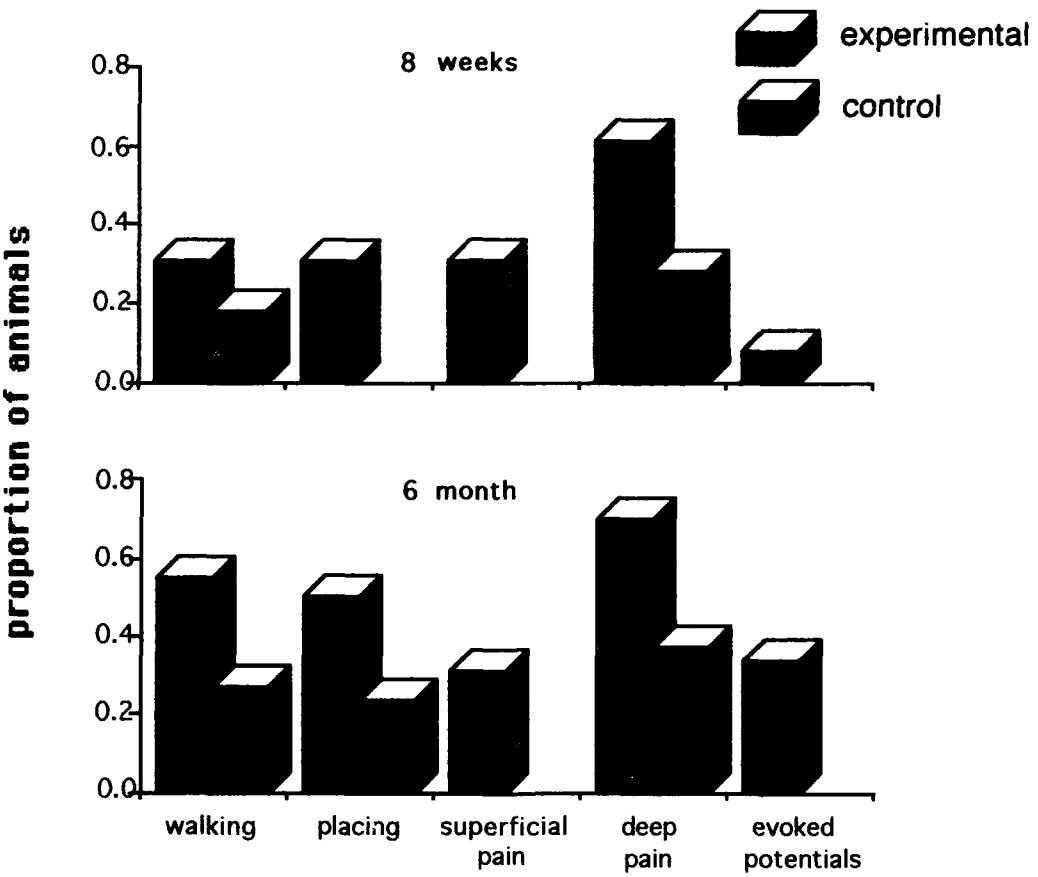


Figure 7

In summary OFS stimulation appears to be an effective technique to accomplish recovery of function in clinical cases of spinal trauma if applied within 1 month of the injury (most convincing results were achieved when the application was within 2

weeks of injury (Borgens et al., 1993b). We were unable to demonstrate any clinically meaningful response to treatment when the application was made at greater times post injury in disc herniation induced spinal trauma or in extremely severe fracture dislocation cases at any time post injury (manuscript in preparation). Further advancement must depend on a deeper understanding of these mechanisms of action at least at *the cell and tissue level of inquiry*.

7. *In Vitro* testing of Astrocyte Responses to Applied DC Fields.

Using a special culture chamber in which an applied voltage could be imposed across populations of GFAP positive cortical astrocytes, we followed their responses to extracellular voltages of 50 - 500 mV/mm by conventional photomicroscopy and time lapse videomicroscopy (Borgens et al., 1994) and a computer derived index of cell Asymmetry (AI) (Hinkle et al., 1981). The cells were derived from primary cultures of rat cerebral cortex, about 80 % of the cultures were determined to be positive for an astrocyte marker (Glial Fibrillary Acidic Protein - GFAP) by immunocytochemical techniques, the applied voltage drop across the chamber, pH, and temperature were continuously monitored, and the extracellular field was imposed using low resistance "salt bridges" so that the cells media was not contaminated by any electrode products generated by electrolysis.

At all field strengths tested, astrocytes immediately resorbed their cellular processes and extended them so that a new bipolar axis of symmetry was achieved such that the cells alignment was perpendicular to the voltage gradient (Figure 8). At field strengths greater than 100 mV/mm this realignment occurred in over 80% of the population (Figure 9). Interestingly, those cells whose orientation was parallel or nearly parallel to the long axis of the applied field prior to field exposure were completely eliminated within hours of exposure to the extracellular voltage gradient. These studies show that the structure and

orientation of astroglia (as well as neurons) is grossly affected by a weak extracellular applied voltage. This further suggests that the organization of the glial "scar" produced by injury to the CNS might be a target of the extracellular voltage - organizing it in such a way as to facilitate - not impede - any potential nerve fiber regeneration (Borgens et al., 1994).

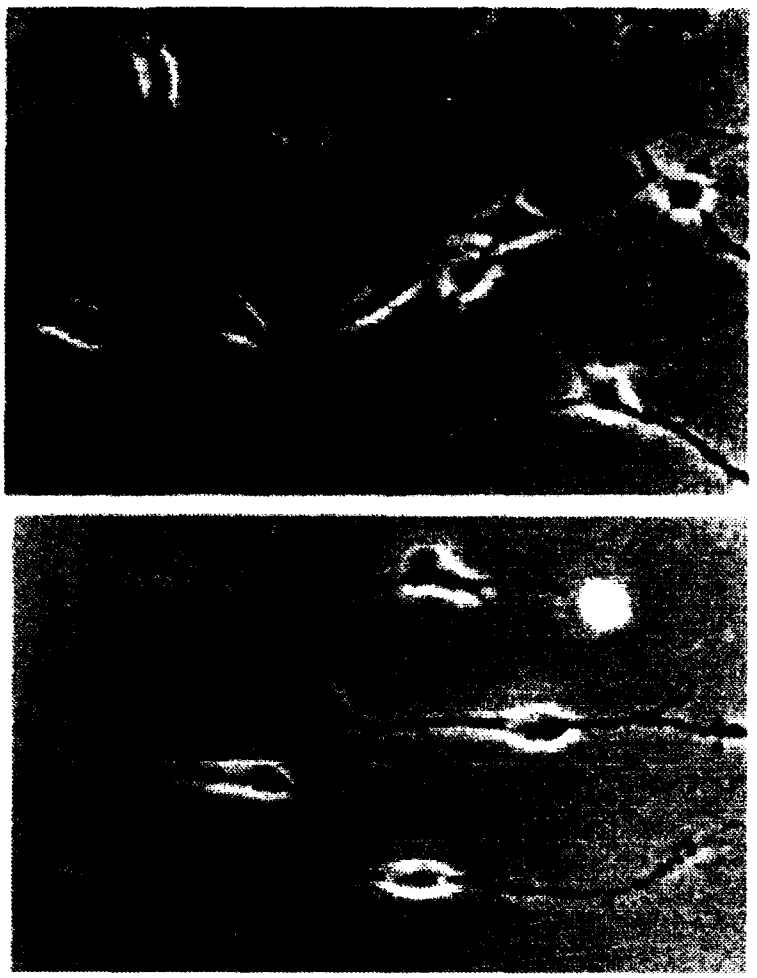


Figure 8

Top: Phasecontrast photomicrograph of astrocytes growing in the experimental chamber prior to electric field exposure

Bottom: 15 hours after current flow from top to bottom (voltage gradient = 320 mV/mm), the cells exhibit a striking perpendicular alignment.

from Borgens et al., 1994

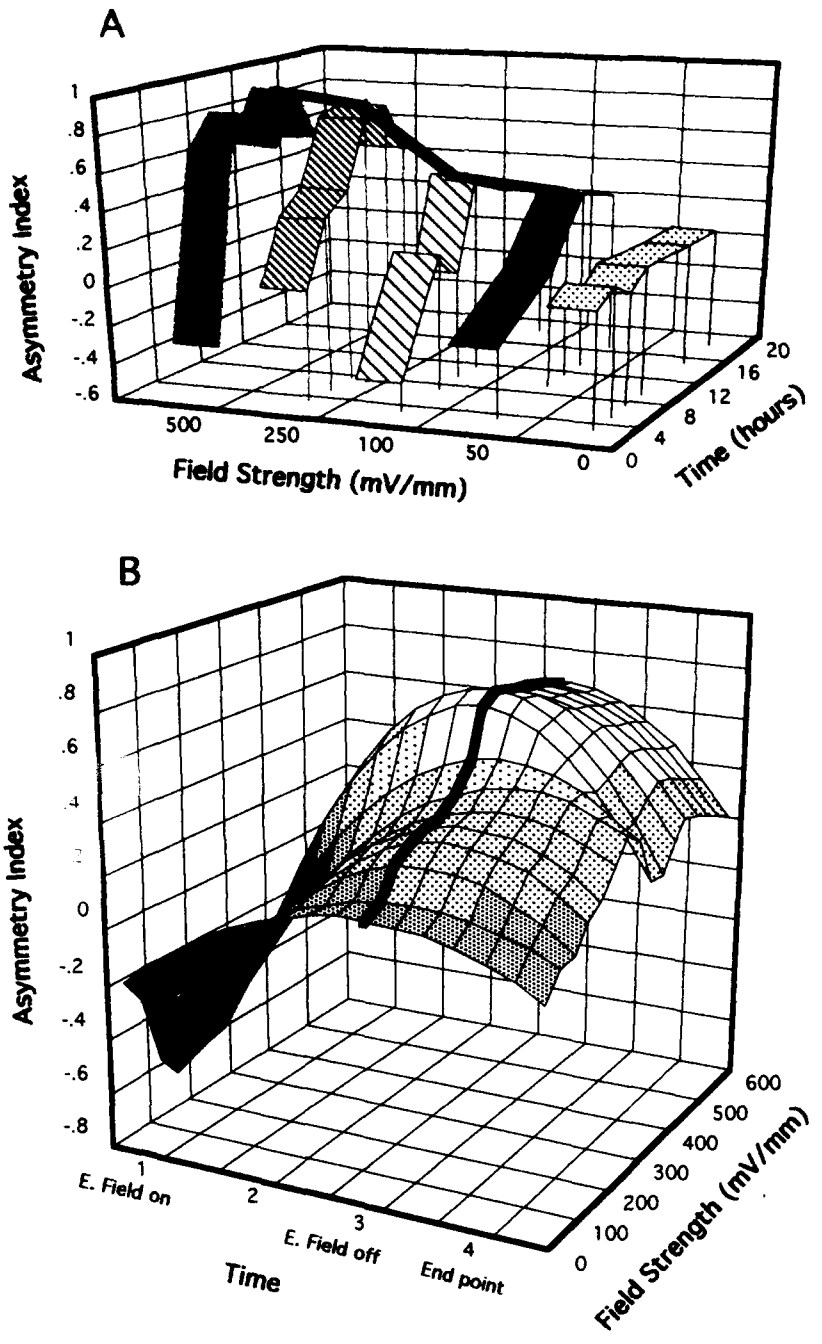


Figure 9

A. The Asymmetry Index (AI) plotted against time and field strength for four representative experiments (50, 100, 250, and 500 mV/mm) and one of two control experiments. An asymmetry index of +1 = all cells within the field are oriented perpendicular to the voltage gradient. An AI of -1 = all cells were aligned parallel to it. Note the marked increase in perpendicular alignment at the higher magnitude of applied voltage. The bold line connecting each plot denotes the point at which the applied field was removed.

B. AI plotted against Field Strength and Time. The AI for all experiments at all field strengths is plotted against 4 time points. The data is normalized around the point of field cessation (bold line as above) and the graph fits the data from the lowest AI calculated (dark gray) to the most perpendicular alignment (white). The values for parallel alignment (negative AI) were not extended to -1 as no values fell below those shown. Note the increase in perpendicular alignment at all field strengths tested, and the fall in AI (trend towards random orientation) following the elimination of the applied field (from Borgens et al., 1994).

CONCLUSIONS

1. Applied DC electric fields are able to facilitate functional recovery in a defined spinal cord injury model (the CTM reflex in rodents) as well as in clinical cases of paraplegia in dogs when applied within one month of the injury.

2. Further delay in the application of the electric field following injury reduces the potential for functional recovery.

3. Studies of neurons *in vitro* and *in vivo,* and more recently astroglia *in vitro,* suggest the behavioral recovery is associated with induced structural changes in these cells - especially oriented regeneration of neuronal processes and possibly reallignment of astroglial processes (which form the CNS cicatrix).

4. The biological sensitivity of neurons and glia to extracellular voltage gradients is related to the fact that a) voltage gradients of this magnitude exist across developing vertebrate neuroepithelia and appear to control their organization during ontogeny, and b) interference in the magnitude or pattern of endogenous extracellular voltage vitiates cranial development.

5) The early application of DC fields across severed or contused nerve fibers also limits retrograde degeneration of axons by limiting the influx of free Ca^{+} into the cellular lesion, when the cathode is distal to the injured tip of the proximal axonal segement.

6) All of the above mentioned effects of applied DC fields are meant to modify the biology of the injury to CNS cells and their processes. We have further developed

autogeneic enteric neuron transplantation (as an alternative to fetal cell transplantation) to possibly allow replacement of neurons lost entirely due to cell death following CNS central hemorrhagic necroses secondary to contusion/transection.

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